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Genes

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13. ABSTRACT (Maximum 200 The overall goal of this project is to identify and characterize the consequences of human mammary epithelial cells (HMEC) that become deficient in normal p53 and BRCA1 gene functions. Our work during the third year of the funding period has produced good progress establishing new systems to create and characterize HMEC with an altered tumor suppressor gene activity. In collaboration with Dr. Bissell's laboratory (UC Berkeley), we have established three-dimensional culture system in our laboratory. This system will allow us to see differences of the growth properties, expression and distribution of certain cell lineage markers, morphology and behavior in between normal cells and partially transformed cells. Our recent acquisition of a Microarray Spotting and Scanning instrument enabled us the use of this robust and sensitive microarray technology to establish genetic expression profiles from any cell source of interest. We have also applied the differential display protocol to identify genes with altered expression in a model system of normal and neoplastic epithelial cells. We firmly believe that these new systems will provide us critical information to our understanding of breast carcinogenesis.				
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FOREWORD

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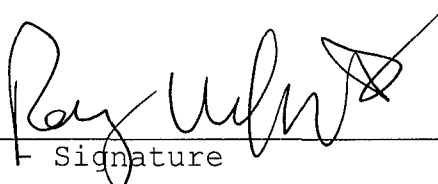

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Table of Contents

Front Cover	1
SF 298, Report Documentation Page	2
Foreword	3
Table of Contents	4
Introduction	5
Body	5
Conclusions	9
References	9
Appendices:	
Tables	12
Figures	13
List of Salaried Personnel	18

(5) INTRODUCTION

Characterization of early events in the development of breast cancer is expected to bring a better understanding of how normal cells are transformed to malignancy and may suggest new strategies for detecting precancerous lesions and new treatment methods. Since germline mutations in two tumor suppressor genes, p53 and BRCA1, are associated with inherited predisposition to cancer (Malkin *et al.* 1990, Hall *et al.* 1990, Hollstein *et al.* 1991, Miki *et al.* 1994, Futreal *et al.* 1994), alterations in the p53 and BRCA1 genes represent some of the earliest genetic changes known to occur in the development of breast cancers.

To study the effects of inactivating mutations in these tumor suppressor genes early in the breast-cancer pathway, we have created p53- and Rb-deficient HMEC by expressing the E6 and/or E7 gene of human papillomavirus type 16 (HPV16). The construction of BRCA1-deficient HMEC has not been achieved yet. The consequences of these genetic changes for cell metabolism will be discovered through controlled *in vitro* comparisons between genetically altered derivatives and their isogenic parent cells.

(6) BODY

The following progress was made during the second year of the funded project (September 22, 1996 to September 21, 1997).

Task 1: Construction of human mammary epithelial cells (HMEC) containing mutations in the p53 or BRCA1 genes.

Our original proposal to construct p53- or BRCA1-deficient HMEC by homologous recombination has been unsuccessful for the same reason described in the 1996 Annual Report. As an alternative, we have created p53- (and Rb-deficient) HMEC by infecting them with retroviral vectors containing the E6 (and/or the E7) gene of HPV16 (1995, 1996 Annual Report). One drawback of using E6 or E7 for creating p53- or Rb-deficient HMEC is that it binds other proteins in addition to p53 and Rb, respectively. To eliminate this possibility, we are currently constructing retroviral vectors carrying the dominant negative mutants of p53 and Rb genes (Dr. Band, Tufts University, personal communication).

Our attempt to down-regulate BRCA1 expression by addition of a BRCA1 antisense oligonucleotide (Thompson *et al.* 1995) to the growth medium (described in our previous report) was not effective. We have found it necessary to implement a more sensitive, reliable and conditional anti-sense gene expression system (described below).

Task 2: Characterization of HMEC containing mutations in the p53 or BRCA1 genes.

Telomerase activation.

In our previous report, we have shown that telomerase was activated in a cell type-specific manner by transduction of HPV16 E6 or E7. Transduction of the HPV-16 E7 gene activates telomerase primarily in early-passage HMEC and transduction of HPV-16 E6 activates telomerase primarily in late-passage HMEC. For both cases, telomerase activation occurs very quickly after transduction, well before immortalization. In order to examine how E7 induces telomerase activity in early-passage HMEC, an E2F-1 transgene was introduced. E7 protein associates with Rb and interferes with its binding to E2F, resulting in impaired Rb cell cycle control functions. As shown in Figure 1, overexpression of the E2F-1 gene induced telomerase, supporting the idea that the relevant

target of the E7 protein is the underlying E7-Rb-E2F pathway. However, it should be noted that overexpression of E2F-1 did not cause immortalization (data not shown), suggesting that telomerase activation at pre-crisis and immortalization may be caused by different underlying mechanisms (manuscript in preparation).

Conditional Expression System

Comparison of test cells expressing a specific protein with control cells without expression requires a sensitive, reliable and conditional (reversible) gene expression system. Several tetracycline-regulated retroviral vectors have been constructed to allow inducible expression of retroviral inserts after integration of the vector in target cells. Unlike the original two-plasmid system, (Gossen et al. 1992, Gossen et al. 1995) the entire system is contained within a single retrovirus (Hoshimaru et al. 1996, Paulus et al. 1996, Yu et al. 1996, Hofmann et al. 1996). In combination with an amphotropic retroviral packaging system, these vectors will provide conditional gene expression quickly and efficiently in a wide variety of cell types, including primary cultures which are usually hard to transfect.

We have acquired four tetracycline-regulated retroviral vectors which allow for conditional suppression (or expression) of cloned genes (LINX, Hoshimaru et al. 1996; SINtet, Hofmann et al. 1996; pRetro Tet-ON and pRetro Tet-OFF, Clontech, Yu et al. 1996, Paulus et al. 1996). To determine the biological characteristics of the first of these vectors, LINX (Hoshimaru et al. 1996), we introduced the Green Fluorescent Protein (GFP) into the vector, produced amphotropic retrovirus using the Amphi-Phoenix packaging cell line (obtained from Dr. Nolan, Stanford University) and infected three different breast cancer cell lines with the construct. The results of these experiments, summarized in Table 1, indicate that the LINX vector can be infected into breast cancer cells with efficiencies in excess of 75%. They also indicate that while GFP is a highly stable protein, the overall concentration steadily declines to minimal levels over a period of four to eight days following transcriptional inhibition by the tetracycline derivative, doxycycline (DOX). We are convinced that a conditional expression system will provide the most clear and convincing data. Please note that the results reported in the table below apply to the induction and repression of our GFP marker in mass culture. We anticipate that we will be able to obtain individual clones that show better levels of both expression and repression. We have constructed vectors containing HPV16 E6/E7 gene and BRCA1 anti-sense gene, and are now testing the conditional expression of cloned genes.

Characterization of HMEC by structure formation assay in Matrigel.

The consequences of mutations in regulatory genes like p53 and BRCA1 genes should be associated with observable cellular and molecular changes. Bissell et al. have shown that culturing breast epithelial cells in an extracellular matrix Matrigel provides a functionally relevant microenvironment conducive to form three-dimensional structures (Weaver et al. 1996, Weaver et al. 1997). Normal cells will form differentiated spheroids with a central lumen. These structures express certain cell lineage markers such as sialomucin at the apical membrane and type IV collagen at the basal membrane. Breast cancer cells, however, are disorganized and lack polarized expression of these markers (Weaver et al. 1996, Weaver et al. 1997). In collaboration with the Bissell laboratory, we have established three-dimensional culture system in our laboratory (Figure 2). We expect to see significant differences, due to the dramatic effect this culture system has on the growth of normal cells and partially transformed cells.

Differential Display.

We originally proposed the *Differential Display* method (Liang and Pardee 1992) to identify genes that are modulated by p53 and BRCA1 deficiency. As our initial experimental model we selected to compare purified normal colonic crypt cells to crypt cells isolated from adenomatous colonic polyps. While the biologic function of breast and colonic epithelial cells differ from each other, the rationale for using colonic crypt cells is firstly based on the fact that the human colonic adenomas are deficient in the APC tumor suppressor protein, which has been shown in mouse to be predisposing for mammary carcinoma as well as colon carcinoma (Moser et al. 1995; Moser et al. 1993). Furthermore, many human mammary tumors have been shown to be deficient in E-cadherin (Kanai et al. 1994; Rimm et al. 1995; Schmutzler et al. 1996), an important cell adhesion protein whose activity is modulated by APC and beta-catenin, each of which are members of the Wnt regulatory pathway (Tao et al. 1996; Vleminckx et al. 1997). It is also worth noting that Wnt-1 is a gene first identified as the target gene for mouse mammary tumor virus in MMTV-induced mouse mammary tumors. As our experimental procedure, we prepared first strand cDNA from total RNA isolated from either normal or adenomatous crypt cells using a collection of twelve anchored poly(T) down-stream primers. Each of the first strand cDNAs thus synthesized subsequently served as template in a PCR reaction against twenty different arbitrarily designed up-stream primers. A total of 240 sample-pairs were prepared in this way and the resulting radiolabeled PCR products visualized in acrylamide gels. To confirm reproducibility of the PCR band patterns all samples were prepared and tested in duplicate (Figure 3). Our Differential Display comparison of the normal and adenomatous colonic crypt cells has been completed, resulting in the identification of at least three genes (CGM2, prothymosin α , and a novel gene with similarity to X box binding protein-1) which consistently and reproducibly show altered expression levels in a panel of two normal and six polyps (Figure 4) (manuscript in preparation). In addition to these three genes more than 30 genes reproducibly displayed altered expression levels in the Differential Display gels, however the very low abundance of these messages in the tissue samples we analyzed prevented us from confirming their exact expression status in traditional methods like Northern Blot analysis and RNase Protection Assay.

Microarray Expression Profiles.

Several new hybridization-based methodologies to quantitatively measure expression levels of large numbers of arrayed genes have been described during the past several years (Fodor et al. 1993; Pease et al. 1994; Schena et al. 1995, DeRisi et al. 1996). While the concept and biochemical principles of this type of analysis are simple, the implementation requires sophisticated and costly robotics and laser-scanning technology that is not readily available. Therefore, to gain access to this technology, our laboratory has established a collaboration with Molecular Dynamics/Amersham, which has developed a full set of instrumentation for creating and reading cDNA microarrays.

The microarray assay is based on a collection of cDNA clones arrayed onto a glass slide (Figure 5). This array is subsequently tested by hybridization against fluorescently-labeled first-strand cDNA generated from tissue mRNA. The strength of the fluorescent signal measured for each of the cDNA clones present on the array correlates to the abundance of that specific mRNA in the tissue. The Molecular Dynamics Microarray Scanner permits the simultaneous detection of two differently-labeled fluorescent probes. The ability to distinguish between two independent fluorescent signals in a single assay allows for direct comparison of gene expression patterns

between two tissues in a single experiment. This feature is particularly useful for experiments where a cell population harboring a conditional expression vector is compared with respect to induced versus suppressed conditions.

The DNA used as the hybridization target on the slides is prepared from selected cDNA clones. We have obtained an initial collection of 1500 unique IMAGE clones, which are arranged in a 96-well microtitre dish format, permitting efficient plasmid purification, PCR amplification and PCR product purification. In our current protocol, a 150µl bacterial culture is grown and lysed using alkaline lysis procedures in a standard round-bottom microtitre dish. Following centrifugation, the plasmid DNA solution is transferred to a Millipore MAHV N45 filter-bottom microtitre dish with 150µl of DNA Purification Resin (Promega). Plasmid DNA eluted from this mini-column method is of high purity and provides an excellent template for PCR amplification of the insert. A second 96-well mini-column purification step separates PCR products from unincorporated nucleotides and primers to ensure that the target DNA performs optimally on the array slide. Prior to deposition on the microarray spotter, target DNA is mixed with a proprietary agent from Amersham. DNA is then bonded to glass slides by UV-cross linking. The microarray spotter is designed to accept the 96-well format and will host eight individual plates per loading. In its current configuration, the spotter can array over 1500 unique DNA targets in duplicate on a single microscope slide. This number will be quadrupled in the next generation of the instrument.

To measure the expression profile (i.e., the relative abundance of individual mRNA species) from a specific cell culture, we will first extract total RNA from the cells using the TriZol reagent. mRNA is isolated from total RNA samples using Qiagen's Oligotex mRNA isolation procedure, then transcribed into first-strand cDNA using AMV reverse transcriptase, following the protocol provided by Amersham. During this process, DNA is labeled by incorporating Cy3 or Cy5 fluorescently-tagged nucleotides at a rate of approximately 1 tag per 100 nucleotides). To minimize any possible bias of the relative abundance of the individual species of mRNA, labeled first-strand cDNA is not further amplified following synthesis. Hybridization takes place under a sealed cover slip in a low buffer volume to achieve the highest possible probe concentration. Once slides are washed and scanned, the expression profile can be established and analysed using ImageQuaNT software, a dedicated computer-based image analysis program developed by Molecular Dynamics.

We are currently using our instruments to analyze gene expression in various breast cells to identify genes displaying altered expression levels among cell lines. Our initial collection of genes for analysis was identified in human colonocytes using a differential display protocol. We have used the microarray technology to confirm that three transcripts whose expression levels vary between normal and adenomatous colonocytes also vary in a comparison between normal and cancerous mammary cells (Figure 6). Following these initial experiments, we have extended the array to include a panel of more than 1500 unique cDNA clones selected to represent various classes of biochemical function likely to be relevant to breast carcinogenesis.

We will use the inducible retroviral system to conditionally express HPV16 E6/E7 and BRCA1 antisense constructs in the primary cultures of HMEC. In each experiment, cell cultures infected with retroviral constructs will be divided and one portion induced with tetracycline. Untransfected cells, with and without tetracycline, will also be carried. Another portion of the infected cultures will be plated in Matrigel for analysis of three-dimensional development. The remaining samples will be lysed in TriZol reagent to prepare total RNA. mRNA will be purified from each sample and to be used for microarray expression profile analysis

(7) CONCLUSIONS

Our work during the third year of the funding period has produced good progress establishing new systems to create and characterize HMEC with altered p53, Rb or BRCA1 gene activity. In collaboration with Dr. Bissell's laboratory (UC Berkeley), we have established three-dimensional culture system in our laboratory. We expect to see differences, due to the dramatic effect this culture system has on the growth of normal cells and partially transformed cells (Weaver et al. 1996, Weaver et al. 1997). Our recent acquisition of a Microarray Spotting and Scanning instrument enabled us the use of this robust and sensitive microarray technology to establish genetic expression profiles from any cell source of interest. We have also applied the Differential Display protocol to identify genes with altered expression in a model system of normal and neoplastic epithelial colonocytes. We firmly believe that these new systems will provide us critical information to our understanding of breast carcinogenesis.

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(9) APPENDICES

Table 1. Doxycyclin (DOX) repression of LINX GFP in infected breast cancer lines as determined by FACS analysis.

To test the efficacy of the tetracycline repressible LINX vector system, GFP was cloned into the LINX vector. Shown here are the results of the addition of DOX to LINX-GFP infected breast cancer cell lines. The effect of DOX suppression is clearly visible after four days of incubation and after eight days incubation, the fluorescent signal is reduced to a small percentage of the original. The GFP signal detected at the eight day mark could be caused either by incomplete suppression of the Tet repressor; alternatively it may be that the signal is residual and results from the slow turn-over rate of the highly stable GFP protein.

Breast Cancer Cell line	Cell-type specific autofluorescence with LINX vector alone	% Cells fluorescent following LINX-GFP infection	Mean Fluorescence Intensity following LINX-GFP infection	Mean Fluorescence Intensity 4 Days after DOX addition	Mean Fluorescence Intensity 8 Days after DOX addition	Residual GFP after eight days of incubation with DOX
MCF7	1.65	76.04	228	90	15	6.6%
BT549	2.02	85.32	629	294	28	4.5%
MDA-MB468	1.82	76.86	599	323	56	9.3%

Figure 1. Telomerase activation by E2F-1 overexpression. (A) Western analysis of cell extracts from early-passage HMEC infected with either vector alone or E2F-1 expressing construct. Total cellular proteins (70 μ g /lane) were separated by 10-20 % gradient polyacrylamide gel electrophoresis and were transferred to nitrocellulose membrane. The E2F-1 protein was detected with anti-E2F-1 (C-20) rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.) and horseradish peroxidase-goat anti-rabbit IgG using an ECL system (Amersham). (B) TRAP assay. The cell extracts were prepared from indicated HMEC after each infection and the amount shown were subjected to the TRAP assay.

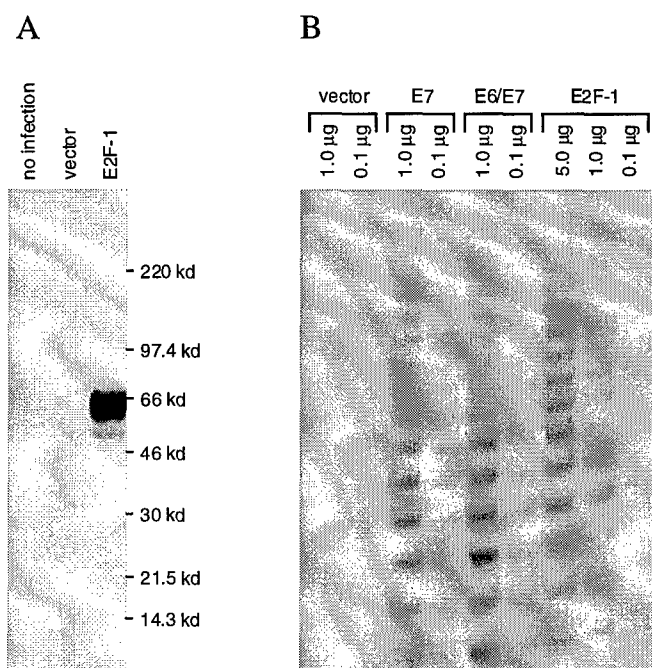
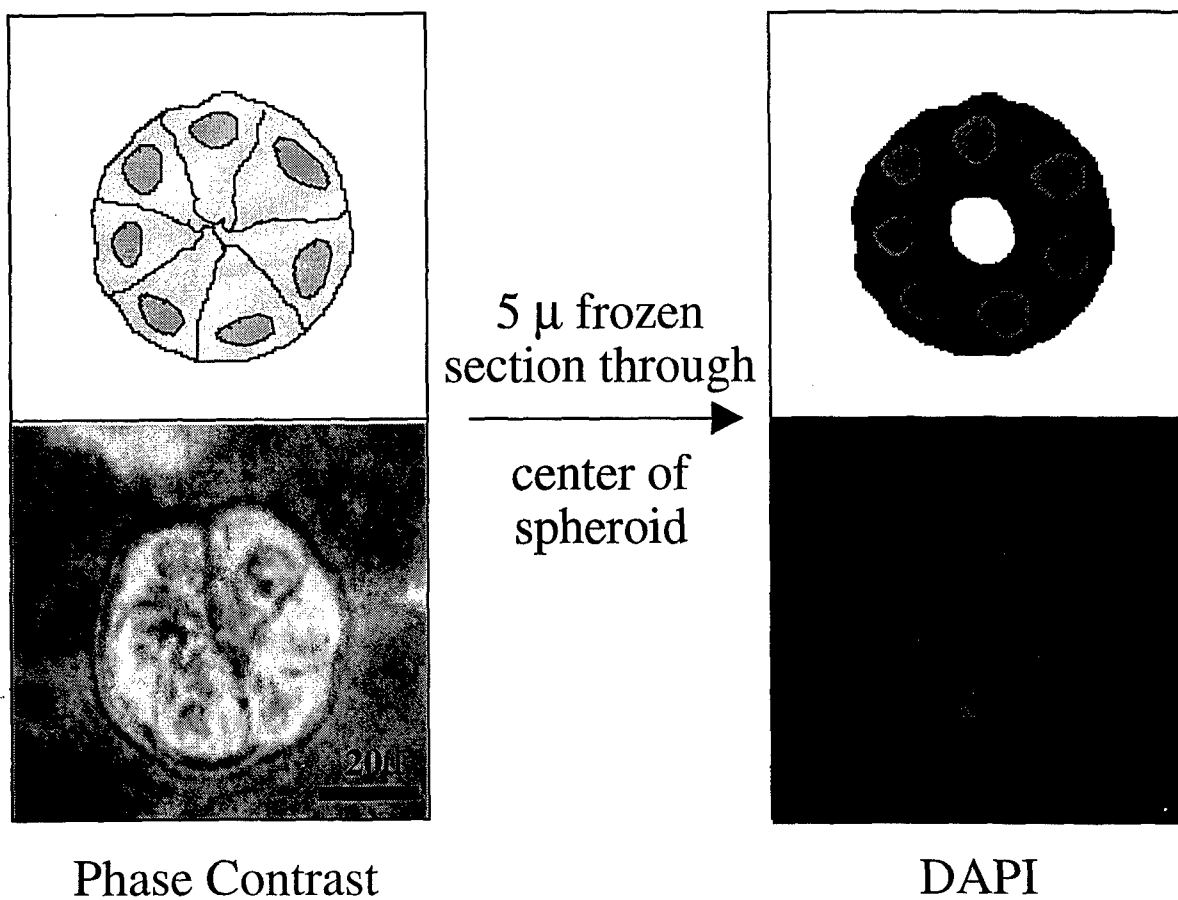


Figure 2. Primary HMEC cultured in Matrigel for 10 days form spheroid structures. Primary HMEC were mixed with Matrigel and cultured for 10 days (Weaver et al. 1996, 1997).



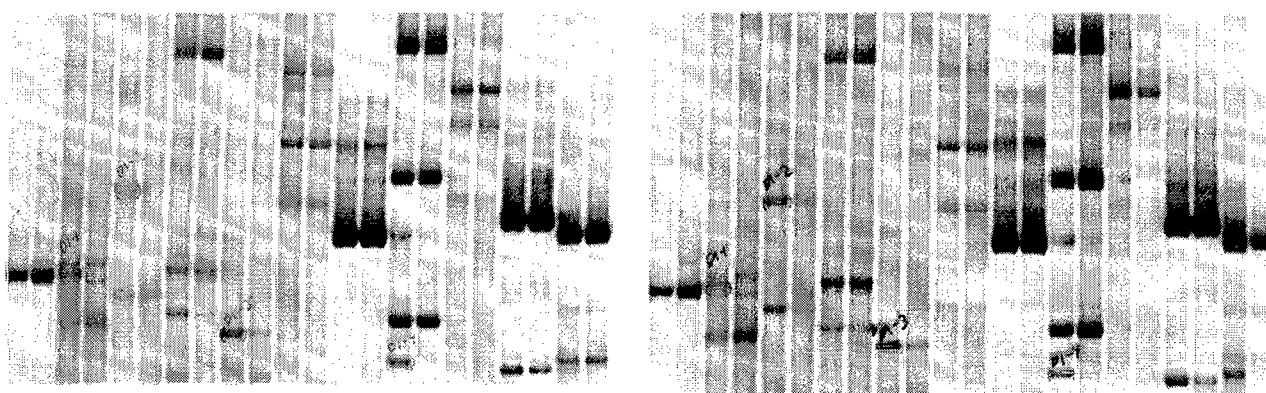
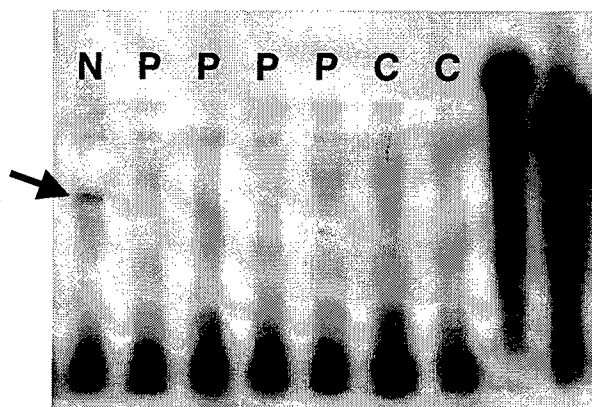


Figure 3. Differential Display Gel. This figure shows eleven paired normal and polyp samples tested with the Differential Display system. An RT-PCR product marked was recovered from the gel and identified as the differentially expressed gene CGM2. Also note the high degree of the high level of identity between paired lanes and between the two independent PCR preparations represented in the left and right panels.

Figure 4. RNase Protection Assay of CGM2.

To confirm the differential expression level of genes identified using the Differential Display system, we compared the expression levels from normal crypt cells (N), adenomatous polyp cells (P) and carcinoma cells (C). A band indicating that the CGM2 gene is expressed only in normal crypt cells and not in the polyp or cancer cells is indicated by the black arrow.



Microarray flow diagram

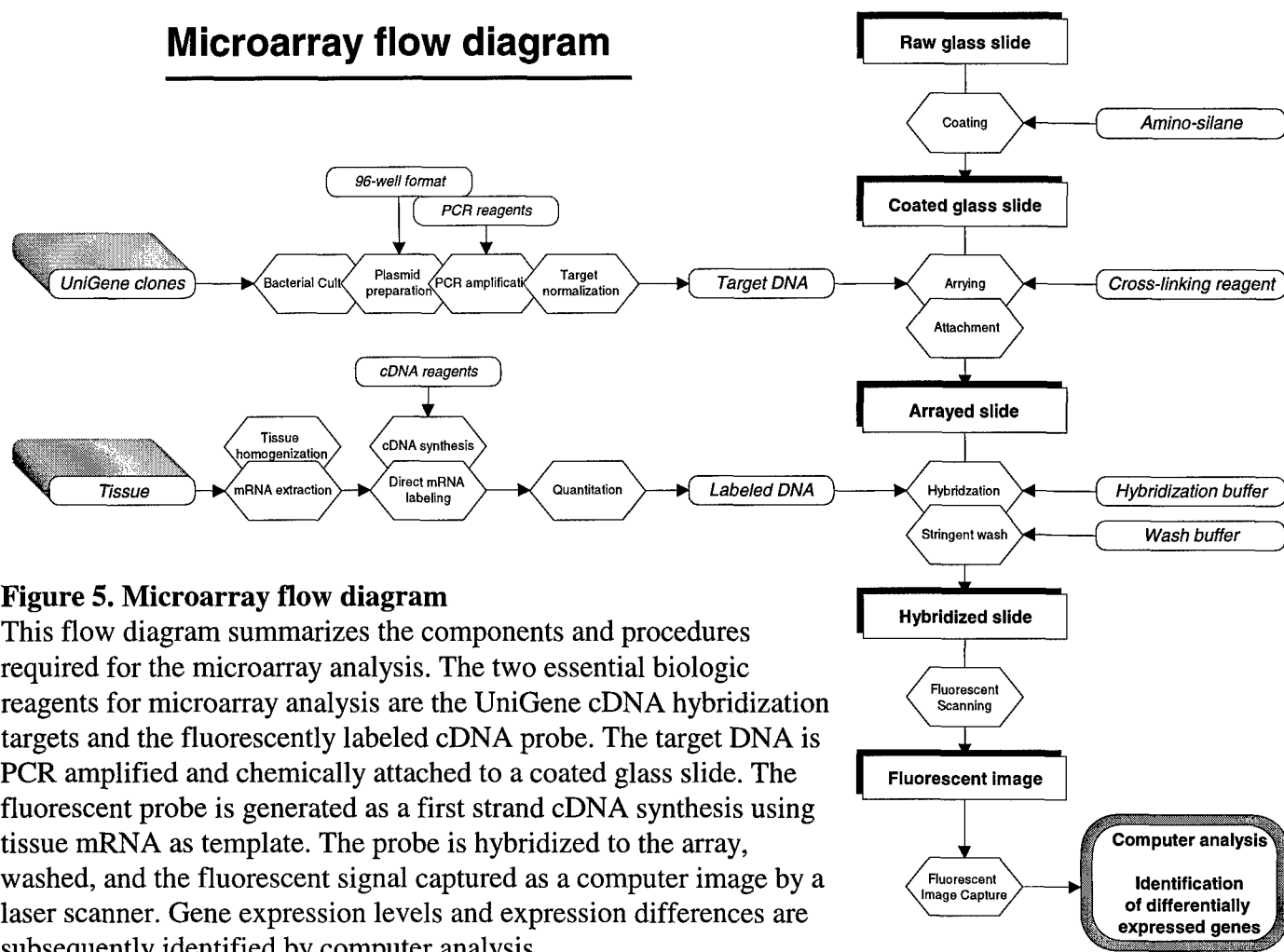
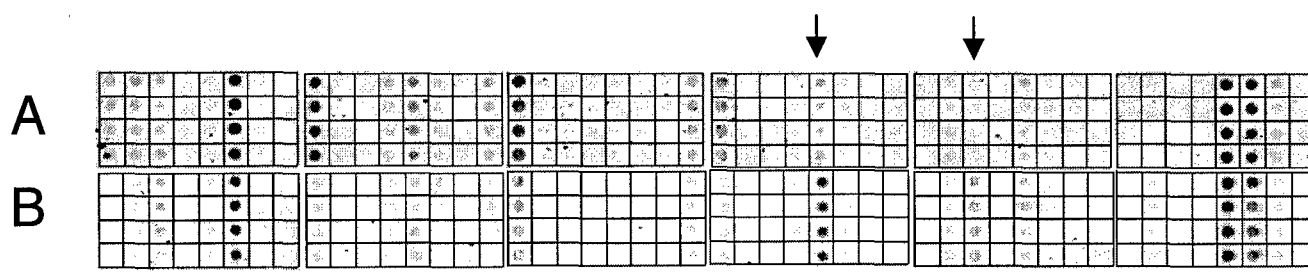


Figure 5. Microarray flow diagram

This flow diagram summarizes the components and procedures required for the microarray analysis. The two essential biologic reagents for microarray analysis are the UniGene cDNA hybridization targets and the fluorescently labeled cDNA probe. The target DNA is PCR amplified and chemically attached to a coated glass slide. The fluorescent probe is generated as a first strand cDNA synthesis using tissue mRNA as template. The probe is hybridized to the array, washed, and the fluorescent signal captured as a computer image by a laser scanner. Gene expression levels and expression differences are subsequently identified by computer analysis.

Figure 6. Microarray Analysis of breast cells BE45 and MDA-MD486.

Panel A is a fluorescent scan of a microarray which was hybridized to fluorescently-labeled first-strand cDNA derived from the normal human mammary epithelial cell line BE45. Panel B is an identical array, which was hybridized to fluorescent cDNA derived from the human breast cancer cell line MDA-MD486. The microarrays contain 47 different cDNA clones and an empty vector (pBS) control. Each column contains a different clone which was spotted four times. The arrows point to two clones, prothymosin- α and a homolog of the X-box-binding protein 1, which reveal different expression levels of the two genes between the two cell types. The two strongest hybridizing clones underlined on the right side of the panel are the housekeeping genes Cyclophilin and GAPDH.



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